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Examining the inhibitory actions of copolypeptides against amyloid fibrillogenesis of bovine insulin

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ABSTRACT

Amyloid fibrillogenesis has been involved in at least 40 different degenerative diseases. The 51-residue polypeptide hormone insulin, which is associated with type II diabetes, has been demonstrated to fibrillate *in vitro*. With bovine insulin as a model, the research presented here examines the influence of two simple, unstructured D,L-lysine-co-glycine (D,L-lys-co-gly) and D,L-lysine-co-L-phenylalanine (D,L-lys-co-phe) copolypeptides, on the *in vitro* fibril formation process of bovine insulin at pH 2.0 and 55 °C. Our results showed that amyloid fibrillogenesis of insulin may be suppressed by both copolypeptides in a concentration-dependent fashion. In addition, the copolypeptides with higher molar fractions of glycine or L-phenylalanine residue, which are considered to possess higher hydrophobic interacting capacities, demonstrated the superior inhibitory potency against insulin fibril formation. Our findings suggest that the association of insulin and copolypeptides, which is likely dominated by hydrophobic interactions and hydrogen bonding, may mitigate the extent of insulin fibrillogenesis. We believe the results from this work may contribute to the understanding of the molecular factors affecting amyloid fibrillation and the molecular mechanism(s) of the interactions between the unstructured polypeptides and amyloid-forming proteins.

1. Introduction

Protein aggregation is a prevalent phenomenon encountered in a broad range of disciplines including biochemical research, biotechnology industry, and human pathology. In general, protein aggregates, including amyloid fibrils with highly ordered structures, and amorphous aggregates with no long-range structure, can be considered as insoluble multimeric proteins that have lost their native forms and functions. Aggregation of proteins is a nuisance in the applications in biopharmaceutical industry where it can interfere with the production and characterization of therapeutic peptides/proteins [1–3]. On the other hand, protein aggregation, especially amyloid fibrillogenesis, is linked to a vast array of debilitating, neuropathic or non-neuropathic human disorders/diseases. Among these are hemodialysis amyloidosis, type II diabetes, Parkinson disease, Huntington's disease, and Alzheimer's disease [4–7]. These diseases display distinct clinical, pathological, and biochemical characteristics, and their corresponding precursor proteins have unrelated functions and share little sequence homology. However, the proteins/peptides that are responsible for these diseases all form similar highly ordered amyloid fibrils exhibiting several morphological and histochemical staining properties in common, for example, exhibition of cross-β structure motif, fibrillar morphology, birefringence upon staining with aromatic dye Congo red, protease-resistance, and insolubility in most solvents [4–6,8–10]. Given the morphological similarities between fibrils that are formed by many different protein building blocks, it has been hypothesized that different proteins follow similar fibril formation pathways [9,11]. However, the detailed molecular mechanisms of amyloid fibril formation remain elusive [5,6,9].

Several non-disease-related proteins/peptides have been induced *in vitro* to self-assemble into amyloid fibrils upon destabilization of their native state [12,13]. Therefore, the fibril-forming propensity polypeptide seems to be largely sequence-independent [14]. Also, the concept that the ability to form amyloid aggregates

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is a basic common property of polypeptide backbones has been widely accepted [9,15]. By taking advantage of the said generic amyloid fibril-forming property, the investigation of the amyloid fibril formation using disease-irrelevant proteins can thus facilitate our understanding of possible mechanism(s) and/or inhibitory action of amyloid aggregation/fibrillogenesis. Bovine pancreatic insulin (BPI), consisting of 51 residues, is a protein hormone involved in regulating glucose metabolism and used to treat diabetes. Its monomeric form consists of a 21-residue A-chain containing one disulfide bond and a 30-residue B-chain, which are linked together by a pair of inter-chain disulfide bonds [16]. Insulin adopts a helix-rich conformation at pH 2.0 (~44% α-helix; ~9% β-sheet, ~30% random coil, and ~19% turn) [17] and exists as a mixture of oligomeric states, including hexamers, dimers, and monomers, in a solution and the composition of which is strongly affected by the environmental conditions [17,18]. The reasoning behind the use of bovine pancreatic insulin as a model in this work is as follows: (1) bovine insulin is one of the best-characterized proteins, and its three dimensional structure, folding-unfolding mechanism, unfolding intermediates, and stability information have been extensively investigated [19–21], (2) bovine insulin is structurally homologous to human insulin (they differ by only three amino acids), which has been found to be responsible for clinical syndrome injection-localized amyloidosis in the human body [22–24] and (3) bovine insulin has been shown to retain a high fibril-forming ability upon incubation in the conditions of acidic pH and elevated temperature [25,26]. As a result, bovine insulin serves as an excellent model system with which to investigate *in vitro* phenomena/behaviors associated with fibrillogenesis.

Several approaches for pathogenic fibrillar and/or aggregated conformers have been put forth, with the attenuation of amyloid fibril formation and/or the capture of fibrillar species being one of the promising avenues for the intervention of amyloid diseases [27–29]. A number of inhibitory molecules, peptidic or non-peptidic, have been shown to suppress the formation of amyloid aggregates both *in vitro* and *in vivo*. Among them, short peptide fragments with sequences mimicking the sequence of amyloid proteins or containing β-breaker amino acids can prevent amyloid aggregation and resultant toxicity due to their marked affinity or molecular-recognition toward amyloid-forming proteins [30–35]. We would like to explore the inhibitory effects of simple random and unstructured copolypeptides on the fibril formation of proteins. Interestingly, findings from our previous study suggest that random copolypeptides may suppress the formation of amyloid fibrils derived by hen egg-white lysozyme (HEWL) [36]. Hence, we were interested in knowing whether these copolypeptides exhibit the *bona fide* inhibitory activity toward amyloid fibril formation. To that end, we employed several spectroscopic techniques, transmission electron microscopy (TEM), and SDS-PAGE electrophoresis, and went ahead to examine the influence of copolypeptides with varying chain compositions (*e.g.*, the ratio of hydrophilic and hydrophobic moieties) and concentrations on the *in vitro* acid-induced fibril formation of an amyloid-forming protein, bovine insulin. We have found that the extent of fibrillogenesis inhibition was correlated with the copolypeptide concentration and composition ratio. The higher molar fraction of glycine or L-phenylalanine residue would result in the better inhibitory potency against amyloid firbil formation by insulin. Our findings suggest that the association or binding between insulin and copolypeptides, which is likely governed by hydrophobic interactions and hydrogen bonding, may interfere with the process of insulin fibrillogenesis. The results from this work may contribute to the understanding of the molecular factors affecting amyloid fibrillation and the molecular mechanism(s) of the interactions between the unstructured polypeptides and amyloid-forming proteins.

2. Materials and methods

2.1. Materials

THF (ACS Reagent, Merck) and diethyl ether (anhydrous, ACS Reagent, J.T. Backer) were dried using Na metal (99.95%, in mineral oil, Aldrich). Hexane (ACS Reagent, ECHO) was dried using calcium hydride (95%, Aldrich). The amino acids used in this work Nε-Z-L-lysine, Nε-Z-D-lysine (99%, Z: carboxybenzyl), glycine (>99%), and L-phenylalanine (>99%) were used as received from Bachem. Bis(1,5-cyclooctadiene) nickel(0) (98t%), 2,20-bipyridyl (99t%), and DMF (anhydrous, ACS Reagent) were used as received from SigmaAldrich, Triphosgene (98%, Merck) was used as received, as were HBr (33 wt% in acetic acid) and NaBH4 (96%) from Fluka. Zinc-free bovine pancreatic insulin was purchased from Sigma (USA), was refrigerated upon receipt and used without further purification. Hydrochloric acid, potassium di-hydrogen phosphate, sodium chloride, and potassium chloride were obtained from Nacalai Tesque, Inc. (Japan). Unless otherwise specified, all other chemicals were purchased from Sigma (USA).

2.2. Preparation of poly(D,L-lysine)-co-polyglycine (D,L-lys-co-gly) and poly(D,L-lysine)-co-poly(L-phenylalanine) (D,L-lys-co-L-phe) random copolypeptides

The nickel initiator 2,2′-bipyridyl-Ni(1,5-cyclooctadiene) (BpyNiCOD) was prepared in a glove box by ligand exchange of the Nibis(COD) complex in the presence of 2,20-bipyridyl in ether. Z-D,L-Lysine, glycine, and L-phenylalanine N-carboxyanhydrides (NCAs) were synthesized using the procedure described by Daly and Poché [37]. Poly(D,L-lysine)-co-polyglycine (D,L-lys-*co*-gly) and poly(D,L-lysine)-co-poly(L-phenylalanine) (D,L-lys-*co*-L-phe) random copolypeptides were synthesized using BpyNiCOD as the initiator [38–40]. The Z protecting group was removed by hydrogen bromide and dialyzed against DI water for three days. The water was exchanged 3–4 times per day. Then the solution was subjected to freeze-drying to remove the solvent and obtain the product as a white spongy material.

2.3. Preparation of fibrillar insulin sample solutions

Sample solutions of 1 mg/mL BPI were prepared by dissolving lyophilized insulin powders in hydrochloric acid with salts (136.7 mM NaCl, 2.68 mM KCl, pH 2.0) and 0.01% (w/v) sodium azide. Insulin sample solutions were first vortexed and then incubated at 55 °C to induce insulin fibrils.

2.4. Thioflavin T fluorescence (ThT) spectroscopy

Amyloid fibril formation was evaluated by fluorescence emission of ThT using a Cary Eclipse Fluorescence Spectrophotometer (Varian, USA). A stock solution of ThT with the concentration determined by the spectrophotometer using a molar extinction coefficient at 416 nm of 26,600 M^{-1} cm^{-1} was prepared in de-ionized water. Bovine insulin samples co-incubated with varying concentrations of D,L-lysine-co-glycine or D,L-lysine-co-L-phenylalanine random copolypeptides (0, 0.5, and 1 mM) were diluted 25-fold with ThT solution (20 μM), and fluorescence intensities were measured by exciting samples at 440 nm and recording emissions at 485 nm.

2.5. Congo red binding assay

An aliquot (160 μL) of the BPI solutions or control buffers was added to 1640 μL of 20 μM Congo red solution in PBS and incubated at room temperature for 30 min before measurement. The spectra